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13. ABSTRACT (Maximum 200 Words)
Primary tumors produced by MCF-7 breast carcinoma cells over-expressing the mostly intracellular human enzyme, exhibited a 3-4 fold accelerated growth compared with tumors produced by mock-transfected cells. A more pronounced increase in tumor size and vascularity was observed with MCF-7 cells over-expressing a secreted form of heparanase, indicating that cellular localization of heparanase plays an important role in breast tumor progression. Translocation, secretion and processing of heparanase were stimulated in response to estrogen. Estrogen also stimulated heparanase promoter activity and gene expression in MCF-7 cells. This effect was abolished by the pure antiestrogen ICI182,780, but not tamoxifen. Our data suggest a mechanism through which estrogen, independent of its proliferative effect, may induce heparanase overexpression and thereby promote tumor-stromal interactions, critical for breast carcinoma progression. The involvement of heparanase in morphogenesis and neoplastic alterations was demonstrated in transgenic mice over-expressing the heparanase enzyme. Mammary glands of these mice exhibited precocious branching and widening of ducts, as well as early signs of hyperplasia and basement membrane disruption. Progress was made in characterization and purification of a proteolytic activity expressed by MDA-435 breast carcinoma cells and involved in processing and activation of latent heparanase. Our results indicate that heterodimer formation between the 8 kDa and 50 kDa heparanase subunits is necessary for heparanase enzymatic activity. Heparanase offers an attractive drug target. Lead heparanase-inhibiting species of laminaran sulfate and chemically modified glycol-split derivatives of heparin were prepared, characterized and found to efficiently inhibit experimental metastasis.

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Introduction

Metastases formation depends on the ability of tumor cells to invade basement membranes (BM) and tissue barriers in a process involving enzymes capable of degrading extracellular matrix (ECM) components (1). While the majority of studies focus on proteolytic enzymes and their inhibitors (2), the involvement of glycosaminoglycan (GAG) degrading enzymes (e.g., heparanase) was underestimated. Despite of earlier reports on the existence of several distinct mammalian HS degrading endoglycosidases (heparanases), the cloning of the same gene (*Hpa*-1) by several groups suggests that mammalian cells express primarily a single dominant functional heparanase (3-5). Preferential expression of the *hpa* mRNA and protein was demonstrated in tissue specimens derived from metastatic melanoma, oral squamous cell carcinoma, hepatocellular carcinoma and carcinomas of the ovary, breast, colon, prostate, bladder, intestine and pancreas (3-5). Patients exhibiting high levels of the *hpa* mRNA had a significantly shorter postoperative survival time than patients whose tumors contained relatively low levels of heparanase (6). There is also a significant correlation between *hpa* mRNA expression levels and tumor vascularity in cancer patients (6).

The overall goal of the proposed research was to study the involvement of heparanase (heparan sulfate degrading endoglycosidase) in breast carcinoma metastasis and angiogenesis. For this purpose, we proposed to determine the effect of increased heparanase expression on breast carcinoma progression in non-metastatic breast cancer cells (task 1). An increased expression of the enzyme was also achieved *in vivo* by producing transgenic mice over-expressing the heparanase gene and protein and analyzing the resulting effect on morphogenesis, hyperplasia and vascularization of the mammary glands. In an attempt to better elucidate the regulation of heparanase gene expression, we proposed to construct a vector composed of the heparanase promoter fused to a luciferase reporter gene and study the effect of estrogenes and anti-estrogens on its expression (task 2). An effect of estrogen on heparanase cellular localization, processing and secretion in breast carcinoma cells was investigated in subsequent experiments. A related aspect was to elucidate the mode of processing and activation of latent heparanase by highly metastatic breast carcinoma cells, toward characterization and purification of the putative heparanase-processing protease (task 1). Finally, we proposed to prepare and characterize heparanase-inhibiting molecules, primarily define species of heparin and laminaran sulfate, and evaluate their effect on heparanase activity *in vitro* and metastasis and angiogenesis *in vivo* (task 3). We have also investigated the inhibitory effect of heparanase specific ribozyme and siRNA.

Body

Task 1. Involvement of heparanase in breast cancer progression

Rationale. *Semi-quantitative RT-PCR was applied to evaluate the expression of the hpa gene by human breast carcinoma cell lines. While the expected PCR product of the hpa cDNA was not detected in non-metastatic MCF-7 breast carcinoma cells, moderate (MDA 231) and highly (MDA 435) metastatic breast carcinoma cell lines exhibited a marked increase in hpa gene expression. The differential pattern of hpa gene expression correlated with the pattern of heparanase activity in vitro (7) and heparanase expression in transplantable tumors induced by these cell lines in nude mice. In subsequent studies, immunostaining for heparanase in breast cancer patients revealed high expression in the in situ and invasive components from ductal and lobular origins (7). Elevated levels of heparanase were found in sera from patients with active breast cancer disease vs. healthy donors, as well as in urine of patients with metastatic breast cancer. There was no activity in the urine of healthy donors (our unpublished results). Collectively, these results provided the basis for our research on the involvement of heparanase in breast cancer progression.*

Task 1a. **Stable expression of heparanase in breast cancer cells and analysis of their tumorigenic properties**

The human heparanase cDNA was subcloned into the eukaryotic expression plasmid pcDNA3 at the *Eco*RI site. Cultured mouse (DA3) and human (MCF-7) breast carcinoma cells were incubated (48-72 h, 37^{o}C) with 1-2 μg *hpa*-DNA and FuGene transfection reagent followed by selection with G418. Stable populations of the transfected cells, evaluated by RT-PCR, were subjected to measurements of heparanase activity. As shown in figure 1, incubation of heparanase transfected DA3 and MCF-7 cells with sulfate labeled ECM resulted in release of low molecular weight heparan sulfate (HS) degradation fragments (peak II, red) into the incubation medium, as opposed to nearly intact heparan sulfate proteoglycans (HSPGs) (peak I, blue) released from the ECM during incubation with the corresponding mock transfected cells. Tumorigenicity of the MCF-7 cells was then evaluated. For this purpose, female athymic nude mice at 6-8 weeks of age were pre-implanted subcutaneously with 1.7 mg of 17 β-estradiol pellets (60-day release). Two groups of mice (10 mice each) were then injected bilaterally into the mammary pads with $1x10^{7}$ MCF-7 cells (pooled population) that were either mock-transfected, or stable transfected with the full length heparanase cDNA. As described above, these cells exhibited little or no vs. a high heparanase activity, respectively. Palpable tumors were felt 4-5 weeks after cell inoculation and their size measured twice a week thereafter. Nine-10 weeks after injection, the mice were sacrificed, the primary tumor removed, weighted, fixed and processed for histological examination. Tumors produced by cells over-expressing the heparanase gene grew faster and reached a 4-5 fold higher size than tumors produced by mock-transfected cells (Fig. 2). Macroscopic examination revealed no visible metastatic colonies. Control studies performed *in vitro* revealed that over-expression of heparanase had no effect on proliferation (doubling time) of the MCF-7 cells. In both cell types, there was a similar stimulation of growth in the presence of low concentrations of estradiol (2×10^{-7}–2×10^{-9} M) and a pronounced anti-proliferative effect at relatively high concentrations (2×10^{-5}M) of estradiol, or Tamoxifen (not shown).

Over-expression of secreted heparanase accelerates breast carcinoma growth and promotes tumor angiogenesis

Rationale. *We have produced a chimeric construct composed of the chicken heparanase signal peptide preceding the human heparanase cDNA (8). Cells transfected with this construct exhibited cell surface localization and secretion of the enzyme, as opposed to a mostly intracellular localization and little or no secretion of the human enzyme (8). Applying both histological analysis of vascular density in sections of the tumor tissue and non-invasive MRI for in vivo mapping of vascular density, maturation and functionality, we have demonstrated a remarkable pro-angiogenic response to cells over-expressing the secreted form of heparanase (9). In comparison, cells expressing the enzyme mostly intracellularly elicited a modest angiogenic response (9).*

Mammary tumors produced by heparanase over-expressing MCF-7 cells grew faster and reached a 3-4 fold higher size than tumors produced by control mock-transfected MCF-7 cells (Fig. 2). In continuation of these experiments, we compared the tumorigenicity of MCF-7 cells over-expressing the human enzyme (H-*hpa*) to that of cells transfected with a secreted form of heparanase (Chimeric *hpa*) (8). Briefly, primary tumors produced by MCF-7 cells expressing the secreted and non-secreted species of heparanase were compared in real time for their vascular functionality and maturation. For this purpose, tumor-bearing mice were subjected to magnetic resonance imaging (MRI) on day 30 after cell inoculation. The size of the primary tumor was higher in mice inoculated with MCF-7 cells expressing the secreted (chimeric *hpa*) vs. the mostly intracellular human heparanase (Fig. 3 A,B). Functionality (VF) and maturation (VD) of the neovasculature were determined from gradient echo images acquired during the inhalation of air, air-CO_2, and oxygen-CO_2 (9,10). The rational for this approach is that vessels coated with pericytes and smooth

muscle cells, in contrast with immature endothelial capillaries, respond to hypercapnia (elevated CO_2) by vasodilatation, while all functional blood vessels show a change in hemoglobin saturation in response to hyperoxia (elevated O_2), reflecting the capacity of erythrocytes to deliver inhaled oxygen to the tumor vasculature (10). Both vasodilatation and changes in hemoglobin saturation are detected by gradient echo imaging, using the intrinsic contrast generated by changes in deoxyhemoglobin, blood volume and blood flow (9,10). As shown in figure 3C, there was a significant elevation in vascular functionality (VF) in tumors produced by MCF-7 cells over-expressing the Chimeric-*hpa* vs. those generated by H-*hpa*- (not shown) or mock- transfected cells (Fig. 3C, VF). Maturation (vasodilatation) of the tumor vasculature appeared to proceed from the margins of the tumor inward and was higher in the center of tumors produced by Chimeric-*hpa*- vs. mock- transfected MCF-7 cells (3C, VD). MCF-7 cells transfected with the chicken heparanase (chk-*hpa*) yielded results similar to those obtained with cells expressing the chimeric enzyme. The high vascularity of tumors produced by MCF-7 cells expressing the secreted (Chimeric-*hpa*) vs. the non-secreted (H-*hpa*) enzyme was also demonstrated by histological examination of the respective tissue sections (Fig. 3D). These results indicate that cell surface expression of heparanase in breast cancer cells markedly promote tumor angiogenesis and maturation.

Task 1b. **Analysis of heparanase proteolytic processing and activation**

Rationale. *The heparanase gene encodes a latent 65 kDa proenzyme that is processed at the N-terminus into a highly active 50 kDa enzyme. The proteolytic cleavage of the 65 kDa proenzyme occurs in two potential cleavage sites, Glu^{109}-Ser^{110} (C1) and Gln^{157}-Lys^{159} (C2) (Fig. 4), yielding an 8 kDa polypeptide at the N-terminus, a 50 kDa polypeptide at the C-terminus and a linker polypeptide of 48 amino acids in between them (11) (Fig. 4, scheme). Proteolytic cleavage is essential for obtaining enzymatically active heparanase, rendering the putative protease a desirable target for drug development. The two cleavage sites (C1 & C2) contain lysines and arginines, but differ from consensus cleavage motives, charcteristic of processing proteases (i.e., metalloproteases, convertases, subtilisins). Moreover, the cleavage at C2 is uncommon as it occurs at the C-terminus of Gln^{157}, suggesting that the protease(s) involved in heparanase processing is unique. We focused on proteolytic cleavage of the Gln^{157}-Lys^{159} (QKK) C2 peptide bond.*

Characterization and purification of protease(s) converting the latent 65 kDa heparanase into a highly active 50 kDa enzyme

We established an assay to measure conversion of the inactive 65 kDa recombinant heparanase into a 50 kDa active enzyme, combining Western blot analysis and heparanase activity assay (Fig. 5A). Serum free medium conditioned by metastatic MDA435 human breast carcinoma cells, as well as cell lysates and plasma membrane preparation derived from these cells, were identified as suitable sources for characterization and purification of the putative heparanase-converting protease. Incubation of the 65 kDa enzyme with each of these preparations yielded excess degradation into low molecular weight peptides (Fig. 5A, lane 2). In an attempt to inhibit this non-specific degradation, BBI (Bowman-Birk soybean serine protease inhibitor) (12) was included in the incubation medium. BBI prevented this undesirable degradation, but had no effect on activation of the latent enzyme (Fig. 5A, lane 3). In order to improve the yield and purity of the processed 50 kDa enzyme, BBI was included during subsequent characterization and purification steps of the MDA-435 derived heparanase-activating protease. Processing and activation was inhibited by the serine protease inhibitors PMSF and DCI, but not by MMP inhibitors. In a search for a phyiological inhibitor of heparanase processing and activation, we have tested the effect of maspin, a member of the serpin superfamily (13), on the ability of MDA-435 cells to convert the heparanase enzyme from a latent 65 kDa form into an active 50 kDa enzyme. A marked inhibition was obtained, suggesting that

the anti-cancerous effect of maspin may be attributed, in part, to its inhibition of heparanase processing and activation (not shown).

i) Purification. MDA435 cell membrane preparation was first subjected to polyethylene glycol precipitation followed by heparin-Sepharose, gelatin-Sepharose and aprotinin-agarose chromatographies. The heparanase-processing protease was not retained on heparin-Sepharose. The unbound material was eluted from aprotinin-agarose with 0.25 M NaCl, yielding a semi-purified highly active preparation (approximate fold purification = 600). In subsequent experiments, peptides corresponding to the potential cleavage region were synthesized and tested for their ability to competitively inhibit the heparanase cleavage reaction. The most active peptide (LREHYQKKFKN) (Fig. 5B, lane 3) was coupled to agarose beads and applied for affinity purification of the heparanase-processing enzyme. An adjacent peptide (FDPKKESTFEERFDER) had no effect (Fig. 5B, line 4) and was applied as a control. Taking into account that the inhibitory peptide may also serve as a candidate substrate, the following flourogenic peptide (amino-benzoic acid-LREHYQKKF(NO_2)KN-NH_2 ; Abz at the N-terminus and p-nitrophenylalanine at position 9) was synthesized and applied as a cleavable substrate for a more convenient characterization and purification of the heparanase-processing enzyme. Our preliminary studies indicate that this substrate is in fact cleaved by the semi-purified MDA-435 derived protease (not shown). We have also coupled the latent recombinant 65 kDa heparanase to Sepharose beads and are now characterizing whether the heparanase processing protease preferentially binds to its latent enzyme target, and hence can be enriched and subjected to Mass-spec and sequence analysis.

ii) Point mutations. Our preliminary studies using peptides corresponding to the two cleavage sites, suggest that processing at C1 and C2 are related. It may be that an endoprotease cleavage at C2 is followed by aminopeptidase that degrades the linker polypeptide in between the two sites in a step-wise manner, until it reaches the C1 end point site. In order to better elucidate whether heparanase processing and activation involve two distinct sites (Fig. 4, scheme), we inserted point mutations in each of the two potential cleavage sites and their adjacent lysines and arginines. The following point mutations were generated: Glu^{109}:Gln; Ser^{110}:Gln; Gln^{157}:Ala; and Lys^{159}:Gln. In addition, Arg^{153}, Lys^{157}, Lys^{158}, and Lys^{161} (at C2) as well as Lys^{107}, Lys^{108} and Arg^{115} (at C1) were replaced by Gln. Cells expressing little or no heparanase activity (MCF-7, JAR choriocarcinoma) were transfected with some of the point mutated full length constructs and tested for heparanase processing (Western blotting) and enzymatic activity. Our preliminary results indicate that Arg^{153} (located 5 residues N-terminal to the C2 cleavage site) is necessary for heparanase processing, since its replacement by Gln abolished processing and activation of the latent enzyme in *hpa*-transfected MCF-7 cells (not shown). Surprisingly, replacement of Gln^{157} and Lys^{158}, had no effect on heparanase processing and activation.

iii) Heterodimer formation. Attempts to express the truncated 50 kDa heparanase protein failed to yield an active enzyme, suggesting that the region N-terminal to the cleavage site is important to the activity of the protein. In fact, the active enzyme is thought to be a heterodimer of the 50 kDa subunit, non-covalently associated with the 8 kDa peptide generated upon processing of the latent enzyme (Fig. 4) (11). We investigated the interaction between the two subunits and its effect on heparanase activity. For this purpose, we transfected MCF-7 breast carcinoma cells with constructs encoding the 50 kDa or 8 kDa proteins, or both. Increased heparanase activity was clearly demonstrated only upon co-expression of the two fragments, indicating that formation of a heterodimer is necessary (Fig. 6, manuscript in preparation).

iv) Involvement of the EGFR system in heparanase processing. Epidermal growth factor receptors (EGFR), primarily the erbB2 variant, are up regulated in a number of human tumors, including breast carcinoma, and are thought to play a pivotal role in cancer progression (14). Indeed, small molecular weight inhibitors of erbB2 signaling, or neutralizing antibodies were effective in xenograft animal models of breast cancer and are currently being applied in the clinic (14). Applying, MDA-435 breast cancer cells, we have noticed that EGFR tyrosine kinase inhibitors (i.e., tyrphostin AG1478) also inhibit heparanase activity in human breast (Fig. 7, bottom) and prostate tumor cell lines. This effect is in all likelihood due to inhibition of heparanase processing, resulting in a reduced amount of the 50 kDa active enzyme and accumulation of the inactive ~65 kDa latent heparanase precursor (Fig. 7, top). Experiments to elucidate the involvement of the EGFR signaling pathway in heparanase processing are underway. Interestingly, our preliminary studies indicate that heparanase expression in biopsies of human breast carcinoma tumors correlates with erbB2 expression (Fig. 7a).

Task 1c. **Inducible expression of heparanase.** MCF7 breast carcinoma cells expressing no metastatic potential and little or no heparanase activity were co-transfected with the heparanase encoding cDNA driven by a tetracycline-responsive CMV promoter (pTET-heparanase) and a vector encoding a transactivator protein (pTET-TAK) (15) that drives heparanase expression only in the absence of doxycycline. Following selection in G418, stable transfected colonies were picked, and tested for tetracycline-regulated expression of heparanase, applying both RT-PCR and heparanase activity measurements. Selected clones expressing a high heparanase activity in the absence of doxycycline vs. little or no activity in its presence (Fig. 8B) were propagated and frozen. Unexpectedly, cells that express high levels of heparanase in the absence of doxycycline, retained a significant activity 3-4 days after its addition (Fig. 8A), suggesting that the heparanase protein is highly stable. Similarly, the *hpa* RT-PCR signal persisted, albeit at a low level, for an extended period of time (3-4 days) after addition of doxycycline into the culture medium (Fig. 8C). Hence, the goal of obtaining a readily regulated expression of heparanase in breast cancer cells was not achieved. The highly stable nature of the heparanase protein was also confirmed by pulse chase experiments, applying metabolically labeled MCF-7 cells (not shown). The tetracycline responsive MCF-7 cells were tested for tumor growth and angiogenesis and found to behave like the above described MCF-7 cells, stable transfected with the human heparanase cDNA (Fig. 2).

Task 1d *(approved after submission of the 1st progress report).* **Involvement of heparanase in branching morphogenesis, vascularization and hyperplasia of the mammary gland**

Rationale. *Cloning of the human heparanase led us to produce transgenic mice over-expressing heparanase. To identify a broad spectrum of phenotypes, we applied the actin promoter to drive over-expression of the heparanase gene in all tissues. Western blot analysis, immunostaining and measurements of heparanase activity revealed a wide spread expression of the heparanase protein in tissues derived from the heparanase transgenic (hpa-tg) mice (not shown). Biochemical analysis of HS isolated from newborn mice and adult tissues revealed a significant decrease in the size of HS chains derived from hpa-tg vs. control mice, reflecting an enhanced heparanase activity (not shown). We applied the hpa-tg system to study involvement of heparanase in mammary gland morphogenesis and potential pre-neoplastic alterations.*

i) Whole mounts. Mammary glands of 3 months old virgin homozygous *hpa*-tg mice were excised and whole mount preparations were prepared for morphological examination. The transgenic mammary glands showed abnormal abundant side branches and precocious alveolar structures, typical of pregnant mice (Fig. 9, top-right). It was also noted that the diameter of many of the mammary ducts was larger in the *hpa*-tg as compared to control mice. Over branching, hyperplasia and widening of glands were best demonstrated in

6-12 month old *hpa*-tg mice (not shown). The same phenotype of extra side branches and wider ducts was noted in heterozygous transgenic mice, but to a lesser extent. The effect of heparanase over-expression on the mammary gland architecture was enhanced during pregnancy (Fig. 9, bottom-right). The alveoli were abnormally clustered and abundant than in mammary glands of control pregnant mice. The ducts were about 5 fold wider in *hpa*-tg vs. control mammary glands, on day 14 of pregnancy.

ii) Tissue sections. The histology of the mammary glands was evaluated in tissue sections. Sections of mammary glands excised from control and *hpa*-tg mice were stained for heparanase, using polyclonal anti-heparanase antibodies recognizing both the human and mouse heparanases. Heparanase was expressed in mammary glands of control pregnant mice to a much lesser extent than in glands removed from the *hpa*-tg mice (not shown). Disruption of the BM surrounding the mammary ducts and alveoli of virgin (not shown) and of 14-day pregnant *hpa*-tg mice (Fig. 10, upper panels) was noted by Masson-trichrom staining of collagen, primarily in areas of side branching. We have also noted a marked increase in vascularization along the ducts and in between the fat cells, in the *hpa*-tg vs. control mice (Fig. 10, lower panels). It appears that an increased heparanase activity in the epithelium of the *hpa*-tg mammary gland is associated with an increased digestion of HS in the BM and ECM, resulting in excess branching and abnormal formation of alveolar structures, as well as widening of the ducts. These effects were enhanced during pregnancy. It is also conceivable that cleavage of BM HSPGs by heparanase unmasks ECM molecules and liberates HS-bound growth promoting factors sequestered by HS in the ECM, resulting in stimulation of cell proliferation and neovascularization. Altogether, our results reflect a pronounced effect of heparanase over-expression on morphogenesis, hyperplasia and vascularization of the mammary gland toward a preneoplastic phenotype, resembling that seen in mice over-expressing stromolysine (16).

iii) Transformation. In subsequent studies, the *hpa*-tg mice were crossed with MMTVneu homozygous mice (Jackson Laboratories). The littermates were divided to neu^+/hpa^+ and neu^+/hpa^- mice, and the mammary glands from both types of progeny were taken for histological evaluation. In neu^+/neu^+ mice, focal mammary tumors begin to appear at 4 months of age, with median incidence of 205 days (17). At two months of age, mammary glands of neu^+/hpa^+ mice showed higher levels of side branches compared to neu^+/hpa^-, suggesting a preneoplastic phenotype of the mammary epithelium in neu^+/hpa^+ mice which may result in tumor development earlier than in neu^+/neu^+ mice. Evaluation of the cross-mated mice for up to a year, failed however to detect mammary tumor formation. Therefore, the *hpa*-tg mice are being backcrossed to obtain a pure Balb/c background that is more favorable for mammary carcinoma development.

iv) Chemical carcinogenesis. Control and *hpa*-tg mice were treated (10-12 weeks) with a chemical carcinogen [i.e., 7,12-Dimethyl-4-aminobiphenyl (DMBA)] known to induce breast cancer tumorigenesis (18). Tumor formation, size, vascularity and invasiveness are being analyzed as a function of time, macroscopically and in histological sections of the tumor tissue, as previously performed in our laboratory in a bladder cancer model (19). Preliminary examination of the mice revealed formation of tumors in about a third of the *hpa*-tg mice vs. none in control mice, indicating that tumor initiation and progression are accelerated by the heparanase-rich tumor microenvironment.

Task 2. **Control of heparanase gene expression, processing and activation**

Rationale. *Given the structural and functional significance of HSPGs in tissue architecture and cell behavior and the potential tissue damage that could result from inadvertent cleavage of heparan sulfate (HS), tight regulation and balance of HS-degrading enzymes (heparanase) are essential, although very little is known at the present time. Of particular interest are parameters affecting hpa-promoter activity, heparanase cell surface expression and secretion, proteolytic processing and activation, cellular uptake and related effects on cell migration, metastasis and angiogenesis.*

Task 2a. **Transcriptional regulation of the heparanase promoter.** The human heparanase gene promoter (1.8 kb in length) was sub-cloned into pGL2 Basic vector (Promega) upstream to the luciferase reporter gene. The activity of the promoter was tested by transient co-transfection of the pGL2 vector together with CMV-LacZ reporter vector. The CMV-LacZ vector has been used to overcome differences caused by different transfection efficiencies. The *hpa*-promoter activity was measured by a luciferase assay kit (Promega) in which an external substrate (luciferin) is added to the lysed transfected cells. In the presence of luciferase activity the substrate is degraded, emitting light (20). Twenty four-48 hours after transient transfection with the promoter construct, the cells are lysed and subjected to luciferase assay. Differences in promoter activity were tested by transfection of the 1.8 kb human *hpa*-promoter into three different cells types: C6 Glioma and MCF-7 breast carcinoma, which normally exhibit very little or no heparanase activity, and MDA-435 breast carcinoma cells, expressing a high heparanase activity. Transfection of C6-Glioma and MCF-7 cells with the *hpa*-promoter resulted in a slight increase in luciferase activity over transfection with the empty pGL2- basic vector (without promoter). In contrast, luciferase activity was highly stimulated following a similar transfection of MDA-435 cells (Fig. 11A). These results suggest that heparanase activity may be regulated at the transcriptional level. Several effector molecules (EGF, bFGF, IL-1, IL-8, LPS, PMA, RA, IFN) that may regulate heparanase activity were tested in MCF-7, MDA-435 and C6-Glioma cells transiently transfected with the 1.8 kb *hpa*-promoter. None of the above agents exerted a significant effect on the heparanase transcription level.

In order to identify regions in the *hpa*-promoter that may regulate heparanase transcription, several deletions were introduced in the 1.8 kb *hpa*-promoter. Each of these deletions (see diagram, Fig. 11B) has been sub-cloned into the luciferase reporter vector, followed by transient transfection into MDA-435 cells. As expected, the highest luciferase activity level was detected by using the full-length promoter (1.8 kb). A 2-fold decrease in luciferase activity was observed when the cells were transfected with fragment #1. Fragments 2-5 yielded a marked decrease in luciferase activity. Fragment # 2 was 15 folds less active compared to fragment # 3. Fragments # 4 and 5 yielded about the same activity as fragment # 3 (Fig. 11B). On the basis of these results it appears that three major regions in the *hpa*-promoter may regulate its activity: region A may act as a positive regulator, increasing (2 fold) fragment # 1 activity. Region B may serve as a positive regulator, markedly increasing the activity of the promoter. Region C may act as a repressor, reducing (15 fold) the activity of fragment #3.

Heparanase promoter methylation. We applied methylation-specific PCR to detect heparanase promoter hypermethylation in human cell lines. High levels of heparanase activity were detected in 15 of the 22 human tumor cell lines studied. While the heparanase promoter was devoid of methylation in the majority of specimens, including MDA435 breast carcinoma, 5 cell lines demonstrated presence of both methylated and unmethylated alleles, and another two (i.e., JAR choriocarcinoma, Hodgkin lymphoma) appeared to harbor only methylated promoters. Remarkably, both of the latter cell lines also lacked heparanase activity. Moreover, none of the 22 cell lines displayed a combination of predominantly methylated promoter region and detectable heparanase activity. Hence, the pattern of methylation status vs. enzymatic activity suggested

an important role for this epigenetic modification in silencing of the heparanase gene. Our results emphasize the potentially increased risk of metastasis following chemotherapy with demethylating drugs such as decitabine and 5-azacytidine (Stepher et. al., manuscript in preparation).

Task 2b. **Estrogen: Effects on heparanase gene expression, cellular localization, processing and secretion**

Rationale. *Although numerous epidemiological evidence clearly suggest that estrogen is one of the main driving forces in breast tumorigenesis, precise mechanisms by which estrogen acts in cancer promotion remain poorly understood. Classically, cancer-promoting action of estrogen has been attributed to its ability to directly promote proliferation of breast cancer cells. However, regulation of cell proliferation is just one aspect of interest in breast cancer research. It becomes increasingly evident that apart of abnormal proliferation, interactions between tumor cells and surrounding stromal components (e.g., enzymatic remodeling and degradation of ECM) are critical for cancer progression, angiogenesis and metastasis (1). Our data indicate that in breast carcinoma, estrogen may affect tumor-stromal interactions through up-regulation of hpa-gene expression (Fig. 12) and degradation of HS in the ECM, affecting neovascularization, local invasion and metastatic spread. We therefore investigated in more detail the regulation of heparanase expression and activity by estrogen.*

i) Transcriptional regulation. Majority of estrogen biological effects are mediated through the classical estrogen receptor (ER)-dependent pathway, where binding of estrogen to nuclear ER leads to the formation of a ligand-receptor complex that binds to the estrogen response element (ERE) in target promoters and up- or down-regulates gene transcription and subsequent tissue response. We have identified three putative estrogen receptor-binding sites in the human *hpa*-promoter, located at positions -1660, -740 and -146 and showing 70-80% homology to the consensus ERE. We constructed a DNA vector in which the *hpa*-promoter region (1.8 kb) containing the detected EREs is introduced in front of a luciferase reporter gene and demonstrated that this region is sufficient to support reporter gene expression in transiently transfected breast cancer cell lines. Transcriptional activity of the *hpa*-promoter was significantly increased in the presence of physiological concentrations of estrogen (1 nM) in ER-positive MCF-7 human breast carcinoma cells (Fig. 12A). Estrogen treatment also induced a 3-4 fold increase in *hpa*-mRNA expression in MCF-7 cells (Fig. 12B), confirming the promoter study data. This effect was abolished in the presence of the pure estrogen antagonist ICI 182,780 (Fig. 12B), but not tamoxifen, the most widely used drug in endocrine therapy of breast cancer. In some experiments, tamoxifen alone even increased the activity of heparanase promoter (not shown). This phenomenon may contribute to endocrine therapy resistance often seen in breast tumors (21) (Elkin et al., manuscript submitted for publication, see appendix).

ii) Angiogenesis. To investigate the involvement of estrogen-driven induction of heparanase in tumor angiogenesis, ER-positive MCF-7 cells were mixed with Matrigel and injected (s.c.) to ovariectomized nude mice. High levels of heparanase were detected in Matrigel-embedded MCF-7 cells injected into mice implanted with estrogen pellets, as compared to mice implanted with placebo. The estrogen-induced increase in heparanase expression correlated with a pronounced angiogenic response, reflected by a well-developed network of capillary blood vessels in Matrigel plugs excised from estrogen-treated mice, vs. little or no vascular response in placebo-treated mice (Fig. 12C) (Elkin et al., manuscript submitted for publication).

iii) Heparanase processing and secretion. Of high significance is the potential regulatory effect of estrogen on heparanase post-translational processing and secretion. We observed that in MCF-7 cells transfected with heparanase under constitutive (estrogen-independent) CMV promoter, treatment with estrogen markedly stimulated secretion of the 50 kDa active enzyme into the culture medium (Fig. 13A). Estrogen also stimulated processing of the latent 65 kDa enzyme into its active 50 kDa form (Fig. 13B). Estrogen may thus affect both *hpa* gene expression and post-translational processing, activation, and secretion, similar to its effect on cathepsin D (22).

Task 2c (new). **Heparanase cellular localization and uptake**

Rationale. *In view of the significance of heparanase cellular localization and secretion on MCF-7 tumor growth and angiogenesis (Fig. 3) and of the effects of estrogen on these parameters (see above), we decided to better characterize heparanase cellular localization and uptake in breast carcinoma cells. These experiments were not emphasized in the original application, but turned out to be clearly relevant to the overall topic of heparanase and breast cancer progression.*

i) Cellular localization. In view of the significance of heparanase cellular localization and secretion, MCF-7 and MDA-231 breast carcinoma cells expressing endogenous heparanase were subjected to transmission electron microscopy (TEM) and immuno-gold labeling of heparanase. Specific heparanase labeling was detected within late endosomes, lysosomes and the Golgi apparatus of these cells (23). A similar distribution pattern was observed in transfected MCF-7 cells over-expressing the heparanase enzyme. Heparanase labeling was also noted in coated pits of MDA-231 cells (23). In other studies, we examined heparanase localization applying immunofluorescence. For this purpose, non-metastatic (MCF-7) and moderately metastatic (MDA-231) human breast carcinoma cells were immunostained with anti-heparanase mAb 130. A perinuclear granular staining was observed in both MCF-7 and MDA-231. Staining of living MCF-7 and MDA-231 cells with LysoTracker yielded the same cytoplasmic granular staining pattern, as also indicated by the co-localization of heparanase and LysoTracker observed in double stained cells (23).

ii) Heparanase activity depends on the integrity of the Golgi apparatus. In order to examine whether heparanase trafficking via the Golgi is essential for its proper maturation, MCF7 and MDA-231 cells were treated with Brefeldin A (BFA), a drug that dissociates the coat proteins from the trans-Golgi membranes, resulting in disassembly of the Golgi. Briefly, cells were preincubated with BFA and examined for their heparanase activity. Untreated cells were used as control. Heparanase activity was reduced (3-5 fold) in MCF7 cells treated with 0.1-10 μg/ml of the drug. A similar inhibition was obtained with MDA-231 cells treated with 1-5 μg/ml BFA. BFA had no inhibitory effect on heparanase activity when incubated directly with purified heparanase, or when BFA treated cells were lysed and incubated with the recombinant enzyme (23).

Our results indicate that following synthesis, heparanase is transported into the Golgi apparatus and subsequently accumulates in a stable form within late endosomes and lysosomes, where it functions in HS turnover. The lysosomal compartment may also serve as a site for heparanase confinement within the cells, limiting its secretion and uncontrolled extracellular activities associated with tumor metastasis, angiogenesis and inflammation (23). Recently, we have noticed nuclear localization of heparanase in cells (human glioma, breast carcinoma) over-expressing the heparanase gene, or cells that were first incubated with exogenous recombinant heparanase. Both the 65 kDa and 50 kDa forms of the enzyme were detected in a nuclear fraction of these cells, as revealed by Western blot analysis and activity measurements. Moreover, some of the enzyme co-localized with HS in the cell nucleus (not shown). The functional significance of heparanase nuclear localization is being investigated.

iii) Cellular uptake and storage. We investigated the interaction of extracellular latent 65 kDa heparanase with primary human fibroblasts devoid of endogenous heparanase. Following heparanase binding to the cell surface and proteolytic cleavage, the processed enzyme is endocytosed and stored within the cells in late endosomes and lysosomes (24). Conversion of the 65 kDa heparanase into the 50 kDa protein was clearly detected (Western blotting) 2-4 h after addition of the latent enzyme to various normal and malignant cells, including MDA435 breast carcinoma (Fig. 4, right). This pattern of events provides a novel regulatory mechanism by which extracellular heparanase, an important source of heparanase activity (released by platelets, inflammatory and tumor cells), is taken up by cells and hence is no longer available for ECM degradation. The enzyme is activated, stored and later on can be utilized to enhance the invasive behavior of cells.

Task 3: Heparanase inhibitory strategies

***Task 3a.* Inhibition of heparanase activity**

i) Laminaran sulfate. Laminaran sulfate (1-3 β-glucan) was found to efficiently inhibit heparanase activity and experimental metastasis in both melanoma and breast carcinoma animal models (25). Because of its low anti-coagulant activity, routine clinical use (in China), and on the basis of our preliminary results, we propose, among other approaches, to use laminaran sulfate (LS) as a starting material for preparation of heparanase-inhibiting oligosaccharides. For this purpose, we applied a systematic enzymatic (laminarase) and chemical methods to produce fragments of LS that were separated (i.e., gel filtration, anion exchange chromatography), purified and characterized in order to determine the minimal length of oligosaccharide and level of sulfation required for efficient inhibition of heparanase activity and experimental metastasis. Fragments of LS containing 12-14 sugar units yielded an almost complete inhibition of heparanase activity at 0.5 μg/ml and of experimental metastasis (B16 melanoma) at 300 μg/ml.

ii) Heparanase-inhibiting species of heparin. The structure of heparin is largely accounted for by sequences of trisulfated disaccharide (TD) units made up of α-linked L-iduronic acid 2-sulfate and D-glucosamine N,6-disulfate, with minor sequences constituted by D-glucuronic and N-acetylated glucosamine residues. Heparin effectively inhibits degradation of HS by heparanase (26). Inhibition of recombinant heparanase was determined for heparin derivatives differing in degrees of N-acetylation, 2-O-sulfation, and glycol-splitting of nonsulfated uronic acid residues (Fig. 14 A,B). We have screened over 40 species of heparin for inhibition of heparanase, using metabolically sulfate labeled ECM as a substrate. Within the unmodified heparin series, the strongest heparanase inhibitors were fractions with the highest content of TD sequences. Partially 2-O-desulfated and glycol split derivatives of heparin inhibited both heparanase activity *in vitro* and experimental metastasis *in vivo* (Fig. 14 C), significantly more than the original heparins. Similarly, glycol splitting of N-acetylated heparin fragment agumented its inhibitory effect, both *in vitro* and *in vivo* (Fig. 14 B,C). Since glycol splitting also involves inactivation of the active site for antithrombin, these derivatives have very low anticoagulant activity. Our results (unpublished) are compatible with a tentative model in which the N-acetylated side of the heparin derivative is recognized by heparanase, and the fully sulfated side strongly binds to the enzyme.

On the basis of structure-activity relationship emerging from the above inhibition studies, we have initiated a collaborative research with Dr. Benito Casu (G. Ronzoni Research Institute, Milan, Italy) aimed to remove excess sulfate groups and improve the molecular flexibility of species of heparin and LS, applying controlled glycol-splitting (27) and sulfation/desulfation strategies. We have already demonstrated that glycol split of nonsulfated uronic acid residues significantly increases the heparanase-inhibiting activity of otherwise unmodified heparins and of heparins 2-O-desulfated up to a total of about 50% of their uronic

residues. Focusing on this approach, we applied progressive N-desulfation/N-acetylation ranging from 10 to 100% of the N-sulfate groups, followed by increasing percentage of glycol-splitting (Fig. 14A). N-desulfation/N-acetylation involved a progressive decrease in inhibitory activity. However, glycol splitting of nonsulfated uronic acid residues significantly increased this activity both for otherwise nonmodified heparins and for N-acetylated heparins, irrespective of the degree of N-acetylation (Fig. 14B). 100% N-acetylated and glycol split (27%) low molecular weight heparin was also an effective inhibitor of experimental metastasis (Fig. 14C). This non-anticoagulant lead compound was found to inhibit angiogenesis in a wound healing model (not shown) and is being tested for an effect on vascularization, primary tumor growth and metastasis of MCF-7 and MDA-435 breast carcinoma cells.

ii) Neutralizing anti-heparanase antibodies. We have produced several monoclonal and polyclonal anti-heparanase antibodies directed against different regions of the molecule. None of these antibodies effectively inhibited heparanase enzymatic activity in our ECM degradation assay. In view of the increasing significance of neutralizing antibodies in cancer therapy (28), we will continue to produce, characterize and test anti-heparanase antibodies, using conventional approaches for generation of site-specific polyclonal and monoclonal anti-heparanase antibodies. Effective neutralizing antibodies will be tested in the MCF-7 and MDA-435 models of breast carcinoma progression.

iii) EGFR inhibitors. EGFR tyrosine kinase inhibitors (e.g., AG-1478; ZD1839) appear to inhibit heparanase processing, resulting in accumulation of the inactive 65 kDa latent heparanase precursor (Fig. 7). Currently, we investigate the ability of a combined treatment with heparanase-, and EGFR-inhibitors to attenuate breast cancer progression. Briefly, the newly characterized heparanase inhibitor (glycol-split N-acetylated low molecular weight heparin) is being tested in combination with EGFR neutralizing antibodies (C-225) or inhibitory molecules (AG-1478; ZD1839) for an effect on primary tumor (*hpa*-transfected MDA-435 and MCF-7 cells) growth, vascularization and dissemination, as described (ref. # 9 & Figs. 2 & 3).

Task 3b (new). **Gene silencing**

i) Ribozyme. We have applied a ribozyme (RNA molecule with sequence-specific endonucleolytic activity) targeting approach to suppress *hpa*-gene expression in breast cancer cells. The heparanase RNA was specifically cleaved by several ribozymes *in vitro*. Cells (MDA-435) transfected with cDNA encoding selected ribozymes showed a markedly reduced heparanase activity *in vitro* and Matrigel invasion *in vivo* (Fig. 15).

ii) siRNA. RNA interference (RNAi) is the mechanism of sequence-specific, post-transcriptional gene silencing initiated by double-stranded RNAs (dsRNA) homologous to the gene being suppressed (29). In mammalian cells, siRNA molecules are capable of specifically silencing gene expression (29). We prepared siRNAs directed against the human and mouse heparanase mRNAs. MDA-435 cells transfected with the relevant pSUPER plasmid (29) exhibited a marked reduction in heparanase transcription (not shown), immunostaining and activity (not shown). *Hpa*-siRNA-transfected MDA-435 cells are being tested for Matrigel invasion *in vitro* and experimental lung and bone colonization *in vivo*.

Key research accomplishments

i) Mammary tumors produced by heparanase over-expressing MCF-7 cells grew faster and were 4-5 fold bigger than tumors produced by control mock-transfected MCF-7 cells.

ii) An inducible (tetracycline responsive) expression of heparanase was obtained in transfected MCF-7 cells. The estimated half-life of both the heparanase mRNA and protein was 3-4 days.

iii) Localization studies, applying immunogold and immunofluoresence staining of heparanase in human breast carcinoma cells, revealed that the enzyme is localized mostly in late endosomes and lysosomes. Integrity of the Golgi apparatus was necessary for heparanase activity.

iv) Cellular localization of heparanase was found to be a major determinant in its involvement in breast cancer progression. Primary tumors produced by MCF-7 human breast carcinoma cells over-expressing a secreted form of heparanase elicited a potent angiogenic response (evaluated by MRI analysis of vascular functionality and maturation) and grew faster then tumors produced by MCF-7 cells expressing primarily the intracellular form of the enzyme.

v) Serum free medium conditioned by MDA-435 breast carcinoma cells was identified as a suitable source for characterization and purification of the putative protease, converting the full-length 65 kDa latent heparanase into an active 50 kDa enzyme. Processing and activation of the latent enzyme by MDA-435 cells was inhibited by serine protease inhibitors (i.e., PMSF, DCI), but not by matrix metalloproteinase (MMP) inhibitors. Maspin, a serine protease inhibitor that partially blocks mammary tumor progression, inhibited processing and activation of the heparanase enzyme.

vi) Specific association between the 8 kDa and 50 kDa heparanase subunits was demonstrated by means of co-immunoprecipitation and pull-down experiments. A region in the 50 kDa heparanase subunit that mediates interaction with the 8 kDa subunit, was identified. Our results clearly indicate that heterodimer formation is necessary and sufficient for heparanase enzymatic activity in mammalian cells.

vii) Transgenic mice over-expressing the heparanase cDNA and protein were generated. Abnormally abundant side branches, precocious alveolar structures and wide ducts were observed in the mammary glands of virgin *hpa*-tg mice. Early signs of hyperplasia and phenotypic transformation were seen in *hpa*-tg mice bred with MMTVneu mice. Mammary glands of pregnant *hpa*-tg mice over-expressing the heparanase enzyme exhibited intense branching of ducts, hyperplasia and basement membrane disruption, associated with abundant vascularization of the mammary tissue. This phenotype reflects the involvement of heparanase in mammary tissue morphogenesis, hyperplasia and vascularization.

viii) The heparanase promoter was sub-cloned upstream of a luciferase reporter gene. A much higher expression was obtained in MDA-435 vs. MCF-7 cells. We identified 3 putative estrogen response elements in the heparanase promoter region and found that heparanase gene expression is up-regulated in response to estrogen in estrogen positive breast cancer cells. Both secretion and processing of heparanase were stimulated in response to pre-treatment with estrogen. These results indicate that estrogen may promote breast cancer progression through stimulation of heparanase promoter activity as well as secretion and processing of the enzyme.

ix) Lead species of laminaran sulfate and N-acetylated-glycol split species of heparin were prepared and found to efficiently inhibit heparanase activity and experimental metastasis. A ribozyme targeting approach was applied to suppress heparanase expression in MDA-435 breast carcinoma cells. A pronounced decrease in heparanase activity and basement membrane invasion was observed in MDA-435 cells transfected with the appropriate ribozyme construct.

x) Our results indicate that heparanase expression, enzymatic activity and involvement in breast cancer progression are tightly regulated by its promoter activity (i.e., estrogen; methylation), proteolytic processing, and cellular localization and secretion.

Reportable outcomes

Manuscripts:

Vlodavsky, I., and Friedmann, Y. Molecular properties and involvement of heparanase in cancer metastasis and angiogenesis. J. Clin. Invest. 108: 341-347, 2001.

Vlodavsky, I., Goldshmidt, O., Zcharia, E., Atzmon, R., Peretz, T., Elkin, M., and Friedmann, Y. Mammalian heparanase: Involvement of heparanase in cancer metastasis, angiogenesis and normal development. Seminars Cell Biol. 12: 121-129, 2002.

Vlodavsky, I., Goldshmidt, O., Zcharia, E., Metzger, S., Peretz, T., Pecker, I., and Friedmann, Y. Molecular properties and involvement of heparanase in cancer progression and normal development. Biochimie, 83: 831-839, 2001.

Goldshmidt, O., Nadav, L., Katz, B-Z. Yacoby-Zeevi, O., Zamir, E., Pecker, I., Geiger, B., Eldor, A., Cohen, I., and Vlodavsky, I. Human heparanase is localized within lysosomes in a stable form. Exptl. Cell Res. 281: 50-62, 2002.

Goldshmidt, O., Zcharia, E., Cohen, M., Aingorn, H., Nadav, L., Cohen, I., Katz, B-Z. Geiger, B., and Vlodavsky, I. Heparanase mediates cell adhesion independent of its enzymatic activity. FASEB J. 17: 1015-1025, 2003.

Presentations:

i) Invited lectures

VIIIth Int. Congress, Metastasis Research Society (London, Sep. 2000)
Glyco XVI (the Hague, Aug. 2001)
53rd Harden Conference "Proteoglycans" (Aug. 2001; Ambleside UK)
ComBio 2001 (Canberra, Australia; October 2001)
Thrombosis & Hemostasis in Cancer (Bergamo, Itali; Nov. 2001)
7th congress of the European Haematology Association (Florence, Italy, June 2002)
2nd International Conference on Tumor Microenvironment (Baden, Austria, June 2002)
3rd Annual Conference on Colorectal Cancer (Montreal, Quebec, September, 2002)
2nd Euroconference on Angiogenesis (Cascais, Portugal, October, 2002)
New Frontiers in Oncology, Israel Cancer Association (Jerusalem, October 2002)
United States – Israel Solidarity Medical Conference (CJP-Hadassah, Jerusalem, November 2002)
BioAcademy Israel 2003 (Tel-Aviv, January, 2003).

ii) Poster presentation: Era of Hope, Department of Defence Breast Cancer Research Program Meeting (Orlando, Florida, September 25-28, 2002); Vlodavsky, I., Zcharia, E., Metzger, S., Goldshmidt, O., Yedovitzky, Y., and Peretz T. Involvement of heparanase in breast cancer progression.

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Yael Friedmann, Ph.D.
Michael Elkin, Ph.D.
Orit Goldshmidt: Ph.D.

Conclusions

Our research focused on each of the approved tasks stated in the original application, toward a better understanding of the involvement of heparanase in breast carcinoma progression. In particularly, we investigated parameters related to the regulation of heparanase expression and activity in breast cancer cells. We identified 3 putative estrogen response elements in the heparanase promoter region and found that heparanase gene expression is up-regulated in response to estrogen at the transcriptional level. As expected, estrogen has no effect on receptor negative cells. Estrogen also stimulated secretion and processing of the heparanase enzyme, similar to its effect on cathepsin D in breast cancer cells. This finding led us to investigate in detail the cellular localization of heparanase, applying immunofluorescence and immunogold staining. We report that the enzyme is localized primarily in late endosomes and lysosomes and that the integrity of the Golgi apparatus is necessary for its enzymatic activity. Nuclear localization of heparanase was also noticed. The mechanism by which estrogen affects heparanase routing, secretion and processing is being investigated. This research is of particular significance in view of our recent *in vivo* studies showing that estrogen promotes angiogenesis and that primary tumors produced by MCF-7 breast carcinoma cells over-expressing the secreted form of heparanase elicit a pro-angiogenic response and grew faster than tumors produced by MCF-7 cells expressing primarily the intracellular form of the enzyme. These results emphasize that translocation of heparanase from the cytoplasm to the cell surface plays an important regulatory role in affecting the involvement of the enzyme in both breast cancer progression and normal morphogenesis of the mammary gland. Tumor angiogenesis was evaluated by MRI analysis of vascular density, functionality and maturation, a non-invasive technique that, in our opinion, is more objective and informative than the previously applied histological examination of the tumor tissue. An intense angiogenic response was also noted in mammary glands of pregnant transgenic mice over-expressing the heparanase enzyme. Another regulatory element that was extensively studied is proteolytic processing and activation of the 65 kDa pro-heparanase by MDA-435 breast cancer cells. We report that this processing is inhibited by potent serine protease inhibitors (i.e., PMSF, DCI), but not by matrix metalloproteinase (MMP) inhibitors. Our results clearly indicate that heparanase processing and activation involve heterodimer formation that is necessary and sufficient for heparanase enzymatic activity in mammalian cells.

To further elucidate the significance of heparanase in breast cancer, we applied a ribozyme targeting approach to suppress heparanase expression in breast carcinoma cells. A marked inhibition of heparanase

activity and basement membrane invasion was observed in MDA-435 cells transfected with the appropriate ribozyme construct. We have also applied the small interfering RNA (siRNAs) approach, using the pSUPER vector for persistent suppression of heparanase gene expression. In continuation of our effort to design heparanase-inhibiting species of oligosaccharides (i.e., heparin, laminaran sulfate) we established a collaborative research with Dr. Benito Casu (G. Ronzoni Research Institute, Milan, Italy) and have already identified lead inhibitory compounds. Studies are underway to better define the minimal length of oligosaccharide and level of sulfation required for efficient inhibition of heparanase activity, tumor angiogenesis and metastasis. In order to further improve the molecular flexibility and biological interactions of heparin, we applied controlled glycol-splitting and sulfation/desulfation strategies. This approach yielded a more efficient inhibition of heparanase activity and experimental metastasis. The effect of selected compounds is being studied *in vivo*, using the heparanase dependent system of breast carcinoma (MCF7, MDA-435) cells over-expressing secreted heparanase.

"So what": Our results clearly support the originally proposed involvement of heparanase in breast carcinoma progression. A conclusive indication is the accelerated angiogenesis and growth of tumors produced by MCF-7 cells over-expressing the human heparanase cDNA and even more so the secreted form of the enzyme. Translocation of heparanase from the cytoplasm to the cell surface and its secretion appear to play an important role in its involvement in breast cancer progression. Progress was made in characterization and purification of a proteolytic activity expressed by breast carcinoma cells and involved in processing and activation of latent heparanase. This protease will serve as a complementary target for identification and development of inhibitory molecules. We have demonstrated that heparanase processing and activation involve heterodimer formation that is necessary for heparanase enzymatic activity in mammalian cells. Lead heparanase-inhibiting species of heparin and laminaran sulfate were prepared, characterized and tested for inhibition of experimental metastasis. We are also applying ribozymes and siRNAs for a persistent suppression of heparanase gene expression both *in vitro* and *in vivo*. Of high significance is the potential regulatory effect of estrogen on heparanase promoter activity, secretion and processing. We have also demonstrated that estrogen promotes an angiogenesis response induced *in vivo* by MCF-7 cells embedded in Matrgel plugs. A potent pro-angiogenic response was observed in mammary glands of mice over-expressing the heparanase gene.

Numerous epidemiological studies indicate that estrogen is one of the main driving forces in breast tumorigenesis, but precise mechanisms of cancer promotion by estrogen remain poorly understood. Classically, tumorigenic effects of estrogen have been attributed to its ability to directly promote the proliferation of breast cancer cells. However, regulation of cell proliferation is just one aspect of interest in breast cancer studies. It becomes increasingly evident that apart of abnormal proliferation, interactions between tumor cells and surrounding stromal components (e.g., enzymatic remodeling and degradation of ECM) are critical for cancer progression, angiogenesis and metastasis. We have found that in breast carcinoma, estrogen may promote these pathological tumor-stromal interactions through up-regulation of heparanase gene expression. Cleavage of HS in the ECM is regarded as a fundamental mechanism of tumor-stromal interaction, neovascularization, local invasion and metastatic spread. The effect of estrogen on heparanase mRNA expression levels was abolished in the presence of the pure anti-estrogen ICI 182,780, but not tamoxifen, the most widely used drug in endocrine therapy of breast cancer. In some experiments tamoxifen alone even increased the activity of heparanase promoter. This phenomenon may contribute to endocrine therapy resistance often seen in breast tumors. Our data suggest a new molecular pathway, through which estrogen, independent of its proliferative effect, may induce heparanase overexpression and thus promote tumor-stromal interactions, critical for breast carcinoma development and progression.

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Figures & Figure Legends

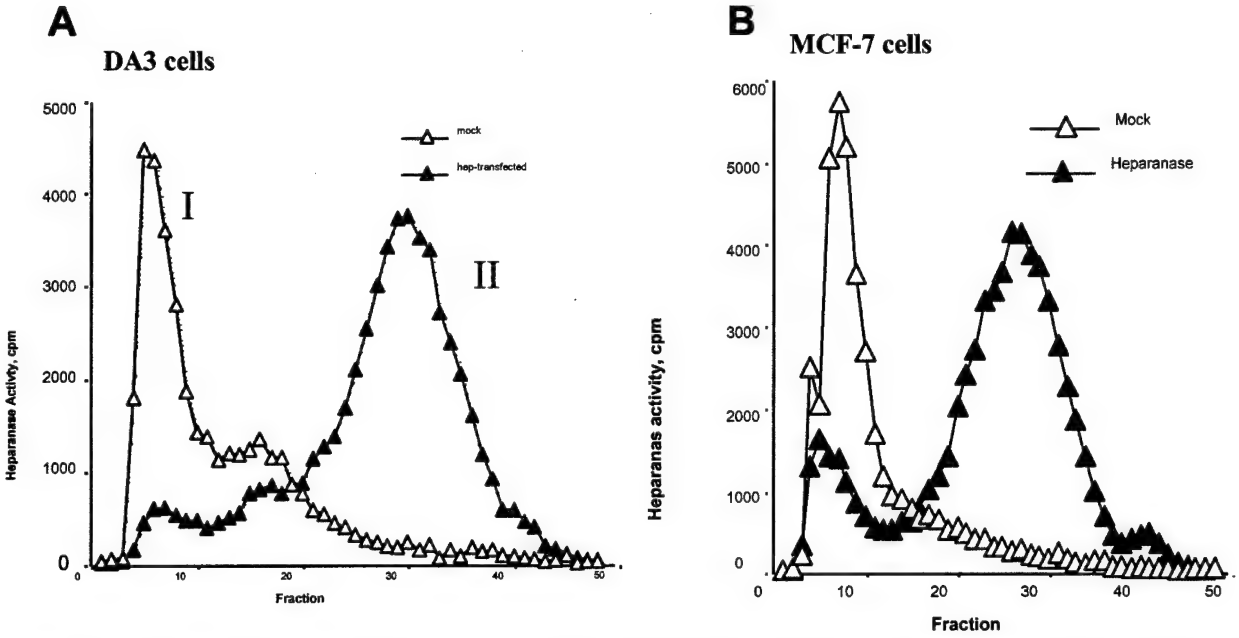


Figure 1. Expression of heparanase activity by transfected breast carcinoma cells. Non metastatic mouse DA3 (A) and human MCF-7 (B) breast carcinoma cells were stable transfected with the human heparanase cDNA (▲), or control plasmid alone (Δ). The cells were incubated with sulfate labeled ECM and labeled degradation products released into the incubation medium were subjected to gel filtration on Sepharose 6B. HS degradation fragments elute in peak II (fractions 20-35).

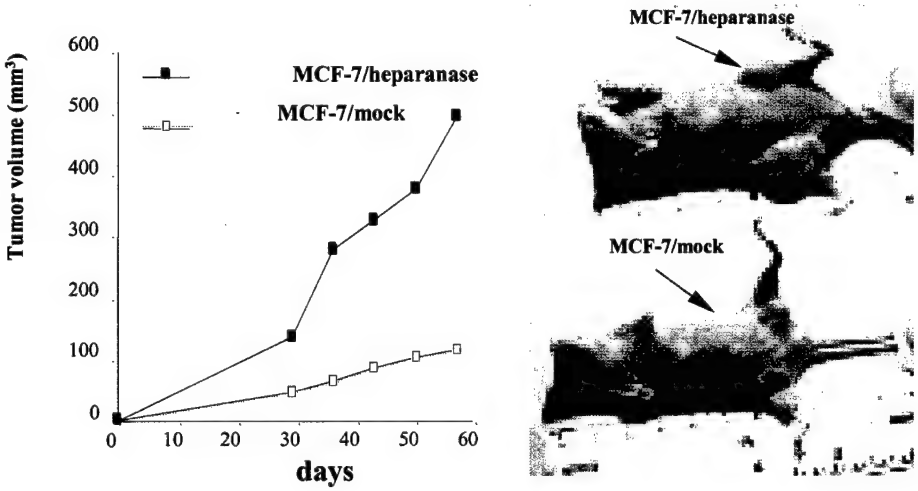


Figure 2. Tumor growth of *hpa*- vs. mock-transfected MCF-7 cells in nude mice. Female athymic nude mice at 6-8 weeks of age were pre-implanted subcutaneously with 1.7 mg of 17 β-estradiol pellets (60-day release). Two groups of mice (10 mice each) were then injected bilaterally into the mammary pads with $1x10^7$ MCF-7 cells (pooled population) that were either mock-, or stable- transfected with the full length heparanase cDNA. **Left:** Tumor size was measured at various times with a caliper. **Right:** Representative mice injected with mock-(bottom) vs. *hpa*- (top) transfected MCF-7 cells.

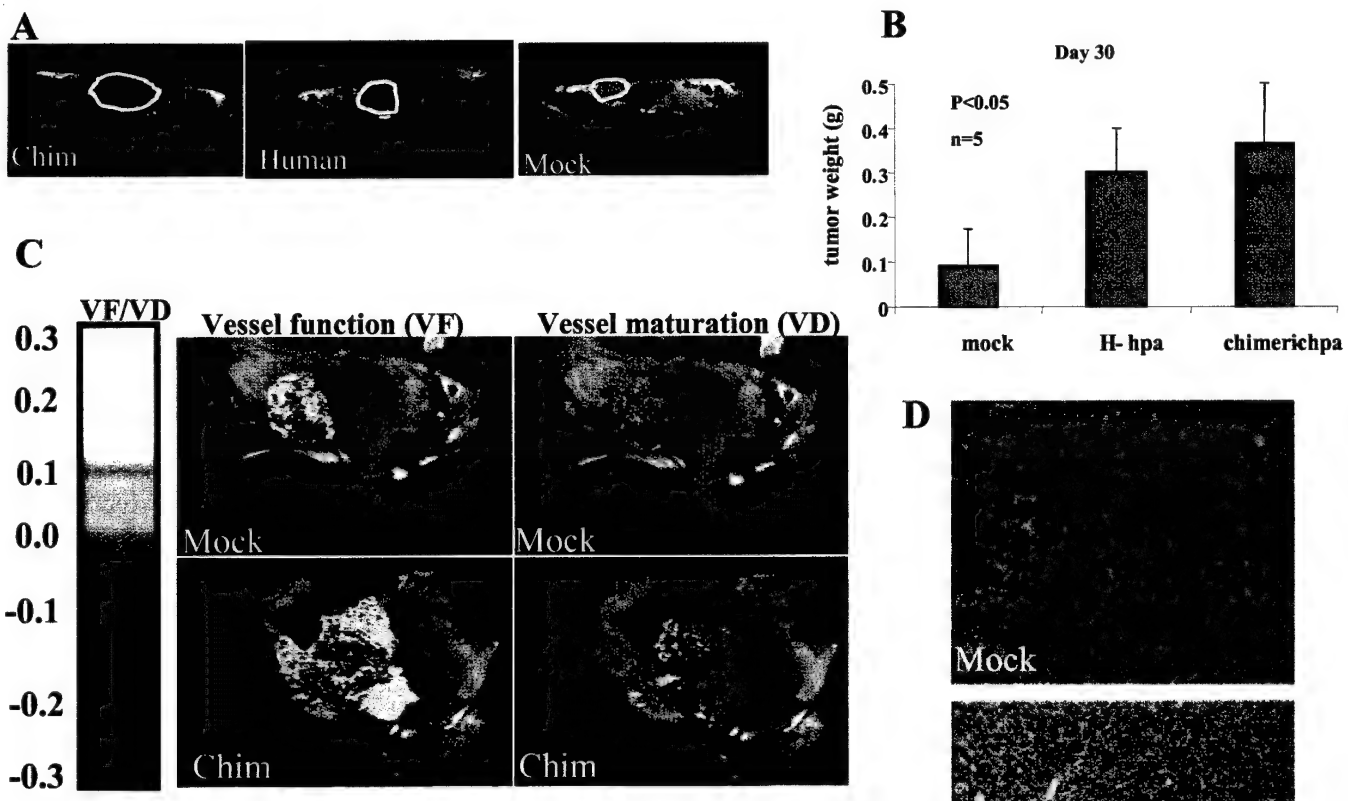


Figure 3. Over-expression of secreted heparanase accelerates tumor growth and promotes tumor angiogenesis. Female CD1 nude mice were inoculated (into the mammary gland) with *hpa*-transfected MCF7 breast carcinoma cells. **A.** Tumor size. Tumor bearing mice were imaged (MRI) on day 30 after cell inoculation. Representative axial gradient echo images of MCF7 tumors produced by Chimeric-*hpa*- (left), Human-*hpa* (middle), or mock (right)-transfected cells. Increased tumor growth is demonstrated (the tumor is marked by a yellow circle). **B.** Tumor weight. MCF7 tumors produced by Chimeric-*hpa*-, Human-*hpa*, or mock- transfected cells were excised on day 30 after cell inoculation and weighed. **C.** MRI analysis of vessel functionality and maturation. Functionality and maturation of the vasculature were derived from gradient echo images acquired during inhalation of air, air-CO2 and carbogen. Color-coded VF and VD maps were derived and overlaid (VF>0.005; VD >0.005, see color scale) on the original coronal images. Note the increased vessel functionality (VF) and maturation (VD) in tumors produced by Chimeric-*hpa*- (bottom) vs. mock- (top) transfected MCF7 cells. **D.** Tumor vascularity. Staining of tumor sections with anti-Von Willebrand (factor VIII) antibody revealed increased vascular density and vessel size in MCF7 tumors produced by Chimeric-*hpa*- (bottom) vs. Human-*hpa* (middle), or mock (top)- transfected cells.

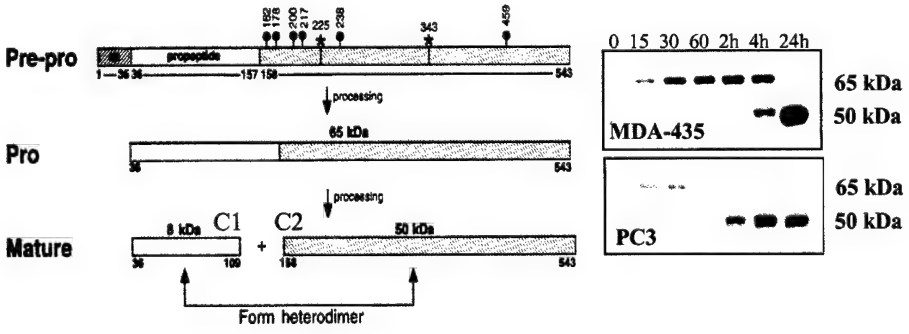
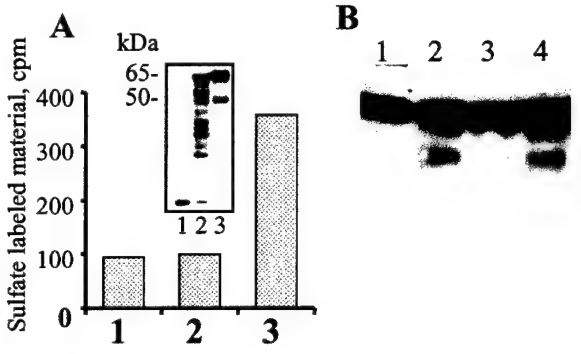


Figure 4. Processing of the 65 kDa latent heparanase into an active 50+8 kDa anzyme. Left: Scheme describing the processing of pre-pro heparanase into mature heparanase, showing the cleavage cites (C1 & C2) and formation of a heterodimer. **Right:** Cellular uptake and processing of exogeneously added 65 kDa heparanase by human breast (MDA-435) and prostate (PC3) carcinoma cells.

Figure 5. Processing and activation of latent heparanase by MDA-435 cells. A. Inset: Western blot analysis (anti-heparanase antibodies mAb130). Cytosol fraction of MDA-435 cells (lane 1) was incubated with recombinant 65 kDa heparanase in the absence (lane 2) or presence of BBI (lane 3). **Bars**: Heparanase activity (release of HS degradation fragments) in the respective systems [i.e., cytosol alone (1), latent enzyme processed in the absence (2) or presence (3) of BBI] was determined using beads conjugated to labeled HS. **B.** Heparanase processing is inhibited by peptide LR...KN (lane 3) but not by FD...ER (lane 4). Lane 1: Latent 65 kDa heparanase; Lane 2: Latent heparanase processed by a partially purified protease produced by MDA-435 cells.



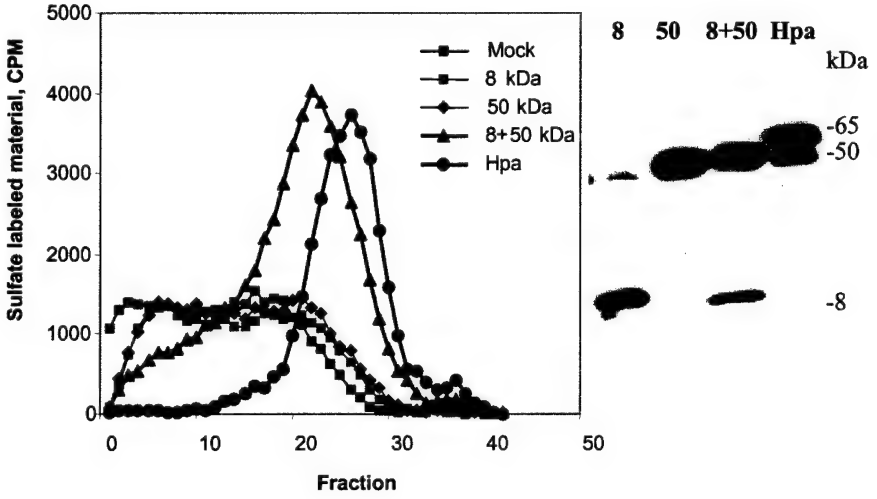
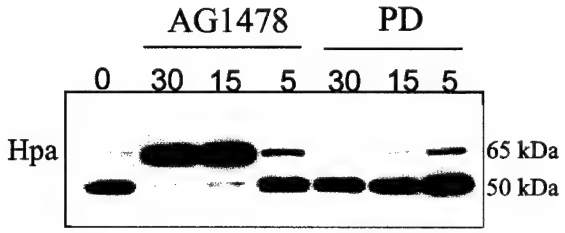
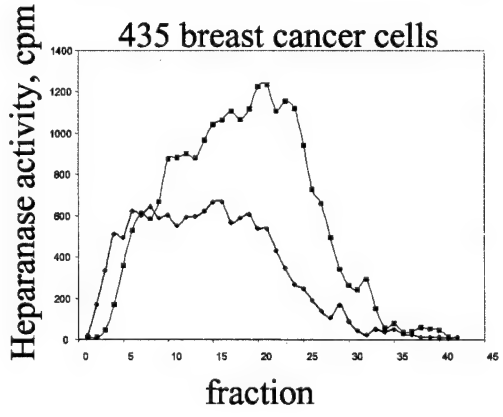


Figure 6. Expression of heparanase activity by cells transfected with heparanase subunits. Left: MCF-7 cells were transfected with constructs encoding the full length 65 kDa heparanase (Hpa), 8 kDa subunit, 50 kDa subunit, or both the 8 kDa and 50 kDa subunits. Mock transfected cells were used as control. Heparanase activity was determined in cell lysates. Sulfate labeled material released from ECM was analyzed by gel filtration (HS degradation fragments are eluted in fractions 15-35). Right: Cell lysates of the same transfected cells were subjected to Western blot analysis applying anti-heparanase antibodies and ECL.

Figure 7. Heparanase processing and activity are inhibited by EGF receptor tyrosine kinase inhibitor. Top: Heparanase processing. MDA435 cells were preincubated (4 h, 37°C) with latent 65 kDa heparanase in the absence and presence of 5, 15 and 30 mM compound AG1478 (an inhibitor of EGFR tyrosine kinase) or PD (an inhibitor of MAP kinase). The cells were then lysed and subjected to Western blot analysis with anti-heparanase antibodies (mAb 130). Processing of latent heparanase into the active 50 kDa enzyme was inhibited by AG1478. **Bottom:** Heparanase activity. MDA435 cells were untreated (■) or pretreated (4 h, 37°C) with 15 mM compound AG1478 (◆). The cells were lysed and tested for heparanase activity, as described in figure # 1.





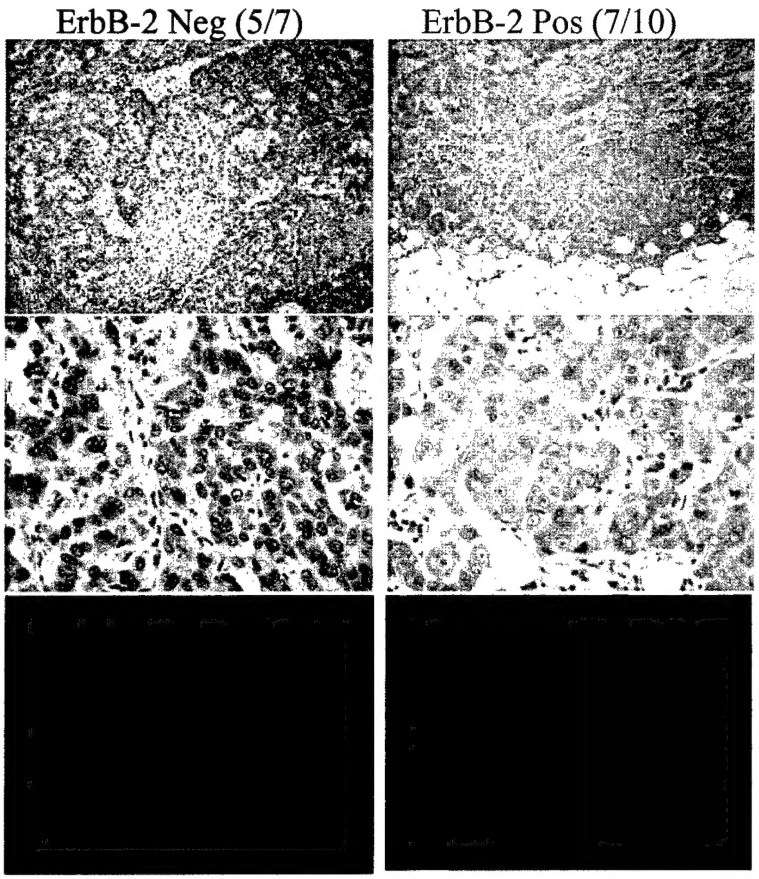
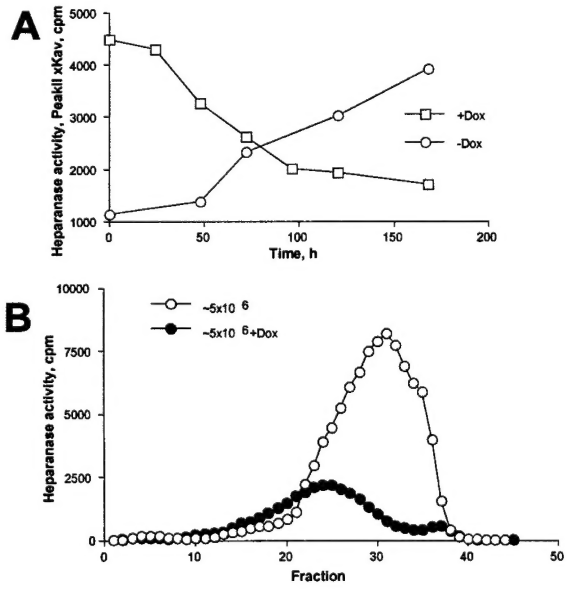


Figure 7a. Heparanase expression correlates with ErbB-2 expression in human breast carcinoma. Tissue sections derived from ErbB-2 negative (left) and positive (right) specimens were stained for heparanase. Intense staining was observed in 7 out of 10 ErbB-2 positive tumors vs. weak staining in 5 out of 7 ErbB-2 negative tumors. Expression of ErbB-2 is seen in the bottom panels.



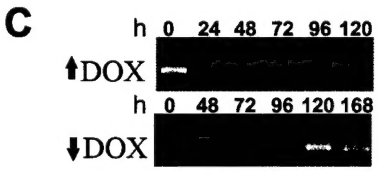


Figure 8. Inducible expression of heparanase. MCF-7 cells were co-transfected with the full-length *hpa*-cDNA driven by a tetracycline-responsive CMV promoter (pTET-heparanase) and a vector encoding a transactivator protein (pTET-TAK) that drives heparanase expression only in the absence of doxycycline. Following selection in G418, stable transfected cells were tested for tetracycline-regulated expression of heparanase, applying heparanase activity (**A & B**), and RT-PCR (**C**). Doxycycline (0.5 μg/ml) was added every other day (□) to cells that were maintained in its absence, or withdrawn (○) from cells that were first maintained in its presence for 3 weeks. At various time periods, cells were tested for heparanase activity (**A & B**), and *hpa*-mRNA expression (RT-PCR). Heparanase activity (release of sulfate labeled HS degradation fragments) expressed by cells maintained in the absence (○) or presence (●) of doxycycline is shown in **B**.

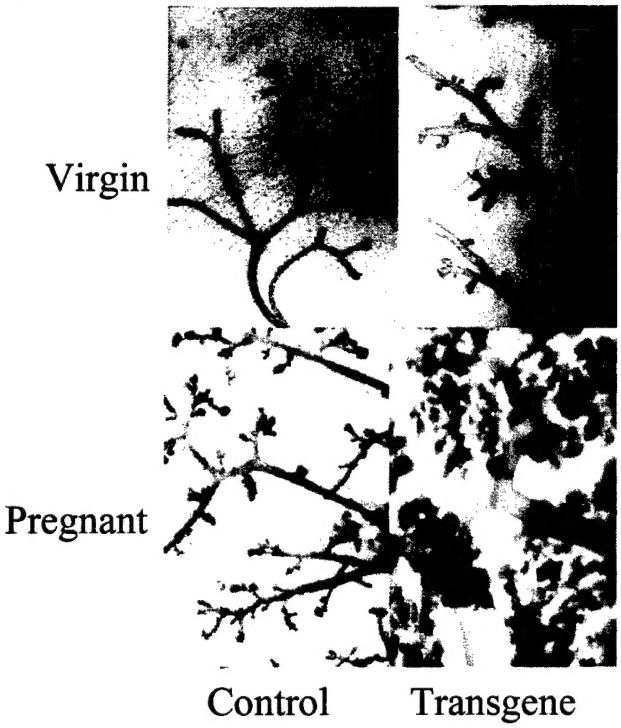


Figure 9. Morphological appearance of mammary glands from control vs. *hpa*-tg mice. Whole mount preparations of mammary glands from 3-month old virgin (top), or 14 days pregnant (bottom) mice were stained with hematoxylin. In both, virgin and pregnant females, the transgenic mammary glands (*right*) showed abundant side branches and alveolar structures, as compared to control glands (*left*). A 3-7 fold increase in the width of ducts was noted in the *hpa*-tg mice.

Figure 10. Histological evaluation of mammary glands of control vs. *hpa*-tg mice. Paraffin sections of mammary glands taken from 14 day pregnant *hpa*-tg (right) and control (left) mice were stained for collagen (upper panels) with Masson-Trichrom.. Abnormal overbranching and clustering of alveoli and excessive disruption of the BM (blue) are seen in the mammary glands of *hpa*-tg mice. This abnormal tissue architecture was accompanied by enhanced vascularization of the transgenic mammary gland (lower panels), as revealed by anti-Von Willibrand immunostaining of vascular endothelial cells. X 200

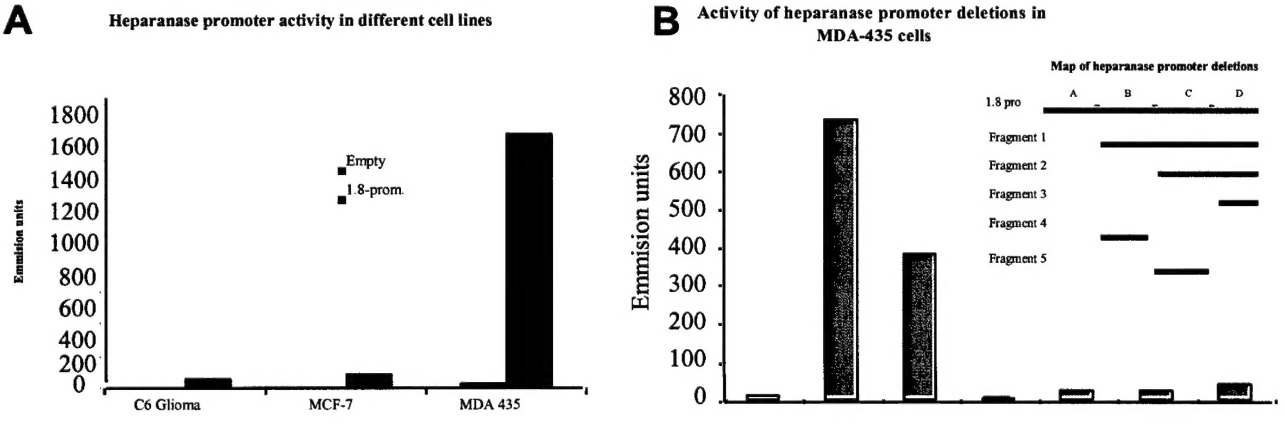


Figure 11. Heparanase promoter activity. A. Luciferase activity expressed by *hpa*-promoter transfected MCF-7 vs. MDA-435 breast carcinoma cells. **B.** MDA-435 cells were transfected with the full-length *hpa*-promoter and with various deleted species of the *hpa*-promoter. Promoter activity (luciferase emission units) was then measured. Inset: Diagramatic presentation of five deleted species of the promoter.

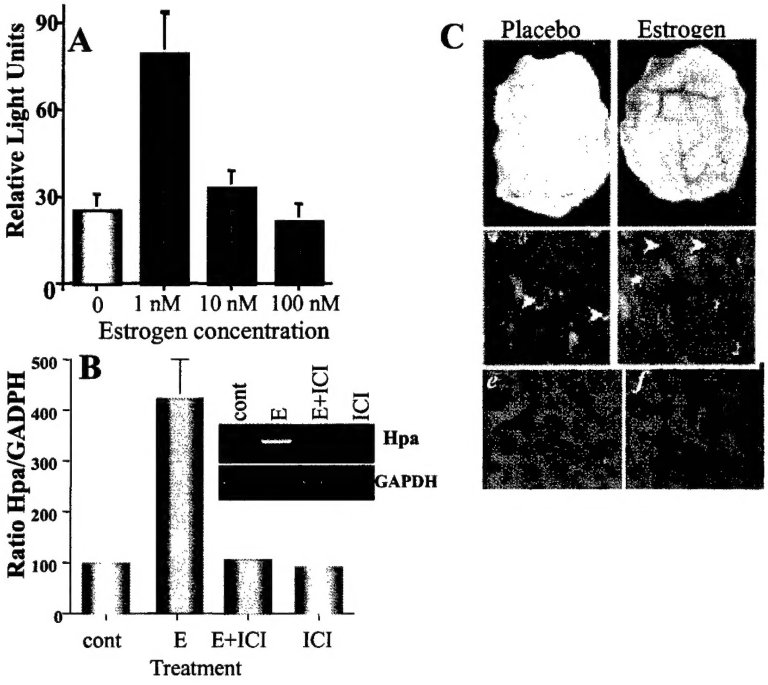


Figure 12. Effect of estrogen. **A.** Physiologic concentrations of estrogen increase hpa-promoter activity in MCF-7 cells transiently transfected with luciferase reporter gene driven by *hpa* promoter. **B.** Estrogen activates heparanase expression in ER-positive breast carcinoma cells. MCF-7 cells were treated (24h) with either 10^{-9} M estrogen (E), 10^{-7} M pure estrogen antagonist ICI182780 (ICI), or both. The estrogen treatment resulted in a ~ 400% increase in *hpa* mRNA levels as quantitated by RT-PCR. Similar results were obtained with another ER-positive T47D cells. **C.** ER positive MCF-7 breast carcinoma cells were mixed with Matrigel and injected s.c. into ovariectomized nude mice, which were previously implanted with either placebo pellet or slow release estrogen pellet. Matrigel plugs were excised 6 days later. ***a*** and ***b***, Matrigel plugs; ***c*** and ***d***, Masson-Trichrome staining; ***e*** and ***f***, immunohistostaining with anti-heparanase antibody of plug sections from animals implanted with placebo (***c, e***) or estrogen slow release pellet (***d, f***). White arrowheads - MCF-7 cells embedded in Matrigel, black arrows - newly formed blood vessels.

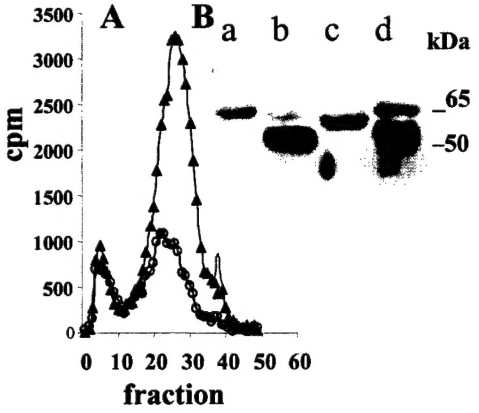


Figure 13. Estrogen regulates secretion and processing of heparanase by breast carcinoma cells. **A.** Secreted heparanase activity. MCF-7 cells transfected with *hpa*-cDNA under constitutive promoter were incubated for 2 h in serum free medium with (▲), or without (○) of 10^{-8} M estrogen. The conditioned medium was then collected and incubated (24 h, pH 6.0, 37°C) with sulfate labeled ECM. Labeled degradation fragments released into the incubation medium were analyzed by gel filtration on Sepharose 6B. **B.** Heparanase processing. Medium conditioned by *hpa*-trancfected MCF-7 cells, cultured in the absence (a, c) or presence (b, d) of 10^{-8} M estrogen, was collected and concentrated on heparin-Sepharose beads. Samples were subjected to Western blot analysis with anti-heparanase antibody. Note that in the absence of estrogen (a, c) only the latent 65 kD form of heparanase is present in the condition medium, while following estrogen treatment (b, d) mostly the processed 50 kDa active enzyme is detected.

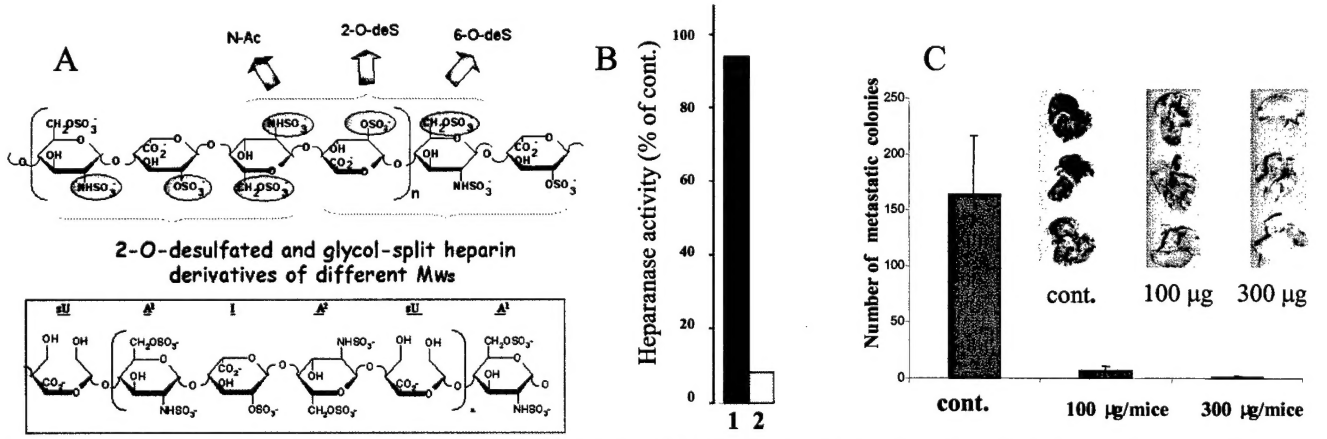


Figure 14. Structure and biological activity of heparanase–inhibiting chemically modified species of heparin. **A.** Low molecular weigh heparin was subjected to N-acetylation, 2-O-desulfation, or 6-O-desulfation (top) followed by glycol-split of the oligosaccharides (bottom). **B.** Heparanase activity is effectively inhibited by N-acetylated & glyco-splitted LMW heparin (2) vs. almost no inhibition by N-acetylated, but not glyco-splitted heparin (1). **C.** C57BL mice received a single i.p injection of PBS containing 0, 100 or 300 µg/mouse N-acetylated & glycol-split low MW heparin, prior to i.v inoculation of B16-BL6 melanoma cells ($1x10^5$ cells/mouse). 15 days later mice were sacrificed, lungs were fixed and examined for the number of melanoma colonies on the lung surface.

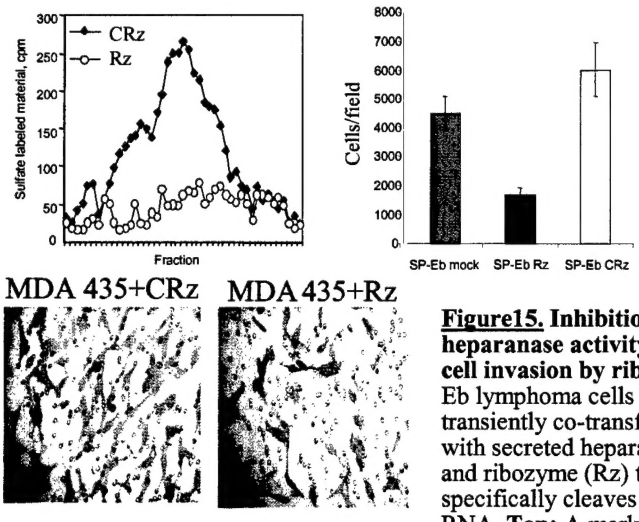


Figure15. Inhibition of heparanase activity and cell invasion by ribozyme. Eb lymphoma cells were transiently co-transfected with secreted heparanase and ribozyme (Rz) that specifically cleaves the *hpa* RNA. **Top:** A marked inhibition of heparanase activity (left) and Matrigel invasion (right) was noted. There was no inhibition in cells transfected with control inactive ribozyme (CRz). **Bottom:** A marked inhibition of Matrigel invasion obtained in MDA-435 cells stable transfected with ribozyme (right, Rz) vs. no inhibition in cells transfected with control ribozyme (left, CRz).